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13. ABSTRACT *(Maximum 200 Words)*

Previously we showed that the transcription of the p53 gene in breast cancer cells was down regulated by OM. To elucidate the molecular mechanisms underlying the OM effect on p53 transcription, in this report, we dissected the p53 promoter region and analyzed the p53 promoter activity in breast tumor cells. We showed that deletion of the 5'-flanking region of the p53 promoter from –426 to –97 did not affect the OM effect. However, further deletion to –40 completely abolished the repressive effect of OM. The p53 promoter region –96 to –41 contains NF-kB and c-myc binding sites, and a newly identified UV-inducible element PE21. Mutations to disrupt NF-kB binding or c-myc binding to the p53 promoter decreased the basal promoter activity without affecting the OM-mediated suppression, whereas mutation at the PE21 motif totally abolished the OM effect. We further demonstrated that insertion of PE21 element upstream of the thymidine kinase minimal promoter generated an OM response analogous to that of the p53 promoter. Finally, we detected the specific binding of a nuclear protein with a molecular mass of 87 kDa to the PE21 motif. Taken together, we demonstrate that OM inhibits the transcription of the p53 gene through the PE21 element. Thus, the PE21 element is functionally involved in p53 transcription regulated by UV-induction and OM suppression.

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INTRODUCTION

Oncostatin M (OM), a 28 kDa glycoprotein, is a cytokine produced by activated T lymphocytes and macrophages (1). Our previous studies showed that OM inhibits the growth of several breast cancer cell lines, including MCF-7, MDA-MB231, and H3922, which is a cell line derived from an infiltrating ductal carcinoma (2-6). Breast cancer cells respond to OM treatment with reduced growth rates and the appearance of differentiated phenotypes. However, OM treatment does not appear to lead to apoptosis. Since the p53 tumor suppressor protein plays important roles in cellular proliferation and differentiation, we examined the effects of OM on p53 expression in breast cancer cells. Surprisingly, we found that p53 expression was down regulated by OM in MCF-7, MDA-MB231, and H3922 cells (7). Decreased levels of p53 protein and mRNA were detected after 1 day of OM treatment and reached maximal suppression of 10-20% of control after 3 days. Nuclear run-on assays further demonstrated that OM decreased the number of actively transcribed p53 mRNA. These studies suggest that OM may repress p53 gene transcription. The effect of OM on p53 transcription appears to precede its effects on cell growth inhibition and induction of morphological changes, as the retardation of cell growth by OM could be only detected after 3-4 days but a decrease in the level of p53 mRNA could be detected as early as 6-8 hours (7). In order to delineate the molecular mechanisms by which OM regulates p53 transcription and to understand the relationship between p53 expression and proliferation and differentiation of breast cancer cells, the Task 1 of my Idea grant is to identify the cis-acting elements and the trans-acting factors that are responsible for the OM-induced suppression of p53 transcription (months 1-18). Therefore, in the past 12 months, we have carried on the investigations to characterize the p53 promoter activity to identify the cis-acting element that mediates the OM effect on p53 transcription in MCF-7 breast cancer cell line.

BODY

Deletion analysis to define the regulatory sequences involved in the basal transcriptional activity of the p53 promoter in breast tumor cells

The regulatory sequences that control p53 transcription in breast tumor cells have not been clearly defined, although a number of studies had examined p53 promoter activity in other cell types. Thus, initially in the present study, we generated a series of reporter constructs in which luciferase gene is driven by varying lengths of the 5'-flanking region of the p53 gene. These constructs were tested for activity in MCF-7 cells. A diagram of the deletion constructs is shown in Figure 1. Figure 2A compared the basal promoter activity of the deletion constructs with the activity of the full promoter construct pGL3-p53Luc that contains a 599 bp fragment of the p53 promoter (-426 to +172) (7). These results, representing 6-8 separate transfections, showed that deletion of the 5'-flanking region from –426 to –177 did not affect the p53 promoter activity, whereas deletion down to –97 (5' Del-4) significantly lowered the basal activity to approximately 40% of the full promoter. Further deletion to –41 (5' Del-5) to eliminate the binding sites for NF-kB and c-myc drastically reduced the basal promoter activity to a level below 5% of the full promoter. These data suggest that the transcription factors NF-kB and c-myc play critical roles in the basal transcriptional activity of the p53 gene in breast tumor cells, however, the promoter region covering –176 to –97 may contain a regulatory sequence that is responsible for the maximal basal transcriptional activity of the p53 gene in MCF-7 cells.

The p53 promoter region from –176 to –97 contains a stretch of CT rich sequence (CCCTCCTCCCC -174 to –164), a potential binding site for the transcription factor Sp1. To determine whether Sp1 interacts with this sequence, electrophoretic mobility shift assay (EMSA) was conducted with the nuclear extract isolated from MCF-7 cells and a ^{32}P labeled double-stranded oligonucleotide, p53-Sp1, corresponding to the promoter region –183 to –154. Upon incubation of p53-Sp1 with nuclear extract, two DNA-protein complexes were detected (Figure 2B, lane 1). Formation of these complexes was inhibited by competition with a 100-fold molar excess of the unlabeled probe p53-Sp1 (lane 2), but was not inhibited by the oligonucleotide p53-mSp1 that contains mutations within the CT-stretch (lane 3). The faster moving complex was supershifted by the anti-Sp3 antibody (lane 5), whereas the slower moving complex was partially supershifted by the anti-Sp1 antibody (lane 4). Inclusion of anti-Sp1 and anti-Sp3 antibodies together in the reaction mixture completely supershifted both complexes (lane 6). These data demonstrate that transcription factors Sp1 and Sp3 bind to this CT-rich region of the p53 promoter.

To determine the function of Sp1/Sp3 in mediating the p53 basal promoter activity, the Sp1 site in p53Luc was mutated (CCCTCCTCCCC to CGCTCGTCGCC) and the mutated reporter p53Luc-mSp1 along with the wild type vector p53Luc were transfected into MCF-7 cells. Figure 2C show that mutation of this Sp1 site lowered the p53 promoter activity by approximately 50%, thereby suggesting that loss of the Sp1/Sp3 binding to the CT-rich region is primarily responsible for the diminished basal promoter activity of the deletion mutant 5' Del-4. These results together demonstrate that Sp1 and Sp3 are positive trans-activators of p53 transcription and that their binding to the CT rich sequence contributes to the basal transcriptional activity of the p53 gene.

Dose-dependent and time-dependent responses of p53 transcription to OM

Next, the effect of OM on p53 promoter activity in MCF-7 cells was examined. The full promoter construct p53Luc was transiently transfected into MCF-7 cells along with the renilla luciferase expression vector pRL-TK. After transfection, cells were untreated or treated with OM at different concentrations for 48 h. Figure 3A shows that the suppressive effect of OM on p53 promoter activity was detected at 0.1 ng/ml, and a maximal suppression of 75-80% of p53 promoter activity was observed at 5 ng/ml. The inhibitory effect of OM on p53 transcription was also time-dependent. The p53 promoter activity was decreased to 67% of control by 8 h, lowered to 35% by 24 h, and further declined to 20% of control by 48 h after treating cells with a saturable concentration of OM. These results clearly demonstrate that OM represses p53 promoter activity in a dose-dependent and a time-dependent manner that is directly correlated with the effects of OM on p53 mRNA expression, as we previously reported (7).

Dissection of the p53 promoter to define the OM-responsive region

To define the OM-responsive region in the p53 promoter, the 5' and 3' deletion constructs of the p53 promoter were transfected into MCF-7 cells. Then the transfected cells were untreated or treated with OM for 40 h prior to cell lysis. The results of 6-8 transfection assays using 5' deletion constructs are summarized in Figure 4. These results showed that deletion of the 5'-flanking region from –426 to –97 did not affect the OM response. In contrast, further deletion to –41 (5' Del-5) eliminated the OM effect. These data suggest that the promoter region covering –96 to –40 is not only important for the basal transcriptional activity as shown in Figure 2A but it may also contain the critical OM-responsive element. Furthermore, shortening of the 3' region from +172 to +14 to delete the PAX binding site had no effect on OM-mediated suppression or the basal promoter activity, thereby excluding the involvement of the repressor PAX in OM-mediated down regulation of p53 transcription (data not shown).

Localization of the OM-responsive sequence to the PE21 element

The 5' deletion analysis localized the OM-responsive sequence to the proximal region of the p53 promoter from –96 to –41. This region contains three important regulatory motifs including NF-kB, the E-box (c-myc), and the newly identified UV-inducible PE21 element. To investigate the role of these regulatory sequences in OM-mediated suppression, site-directed mutagenesis was conducted on the full promoter p53Luc to mutate each binding site individually. Figure 5 shows that mutation of the c-myc site lowered the basal promoter activity 75% without affecting the OM effect. Likewise, mutation of the NF-kB binding site decreased the basal promoter activity 85% with little effect on OM. By contrast, mutation at the PE21 element drastically reduced the basal promoter activity and rendered the p53 promoter unresponsive to OM. To confirm this finding, the PE21 element was mutated in the vector 5'Del-4 that contains the minimal sequence for the basal transcription and the OM-mediated suppression. Again, the OM inhibitory effect was not observed in the PE21 mutant in the context of this short promoter fragment. Similarly, deletion of the PE21 motif from the 5' Del-4 (5' Del-4b) resulted in the loss of the OM response.

Next, we were interested in determining whether OM could exert its effect on PE21 in the context of a heterologous promoter that contains the PE21 element without auxiliary sequences of p53 promoter. To test this, luciferase reporters containing different copies of PE21 in tandem inserted 5' upstream of a minimal HSV tk promoter

(pTKLuc) in either sense, pTKLuc-PE21 (S), or antisense, pTKLuc-PE21 (As) orientations were transfected into MCF-7 cells. The plasmid pTKLuc produced low but measurable luciferase activity in MCF-7 cells and OM treatment did not lower the activity. Inclusion of the PE21 sequence in either direction greatly increased luciferase activities from 20-fold to 400-fold of the pTKLuc. The fold increase of luciferase activity was concurrent with the increase in PE21 copy number in most cases and showed a preference with the antisense orientation. Importantly, in contrast to the vector pTKLuc, the reporters containing the PE21 element clearly displayed responses to OM with luciferase activities reduced to 39% to 59% of control in the OM treated cells, comparable to the OM effect observed in the native p53 promoter. Together, our results presented in Figures 5 and 6 provide strong evidence that the PE21 element plays an important role in the basal transcription of the p53 gene and it is also critically involved in the OM-induced transcriptional suppression of the p53 gene in breast cancer cells.

Characterization of nuclear proteins that interact with the PE21 element

To detect nuclear proteins in MCF-7 cells that specifically interact with the PE21 sequence, EMSA was conducted with ^{32}P-labeled oligonucleotide p53-PE21 containing the PE21 and flanking sequence, and nuclear extracts prepared from untreated or OM-treated cells. Figure 7 shows that 3 specific complexes were detected in both control and OM-treated nuclear extracts. The formation of these complexes was inhibited with 100x-fold molar excess of unlabeled probe (lanes 2, 7) but was not inhibited by a 100x-fold molar excess of an unrelated DNA containing an estrogen response element (lanes 5, 10). An oligonucleotide containing the NF-kB site of the p53 promoter competed for the binding of complex A but not complexes B and C (lanes 4, 9). The binding of complex A was also competed by oligonucleotide p53-mPE21 that contains a 3 bp mutation within the PE21 sequence (lanes 3, 8). Since the PE21 probe covers the positions –82 to –56 including the 5' end of the NF-kB site, we believe that complex A contains subunits of nuclear factor NF-kB.

Complexes B and C are PE21 specific as the oligonucleotide p53-mPE21 lost the ability to compete with the binding of these two complexes to the labeled PE21 probe. Apparently OM treatment did not altered the pattern of the complexes or the intensity of the binding signals. This observation was consistent with the results of UV-induction. It was shown that the binding of nuclear proteins of fibroblasts without or with UV-irradiation to the PE21 probe was not different (8).

Previous studies conducted in human fibroblasts did not characterize the protein/DNA complex of the PE21 sequence. It is unknown whether a single DNA binding protein or multiple proteins interact with the PE21 motif. To characterize the MCF-7 nuclear proteins present in the PE21 DNA complexes, EMSA was followed by UV cross-linking. Complex B was excised from the gel and the protein components were analyzed by denaturing SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Analysis of the SDS-PAGE revealed that one protein was crosslinked to the labeled PE21 probe (Figure 8). After correction for the bound oligonucleotide, the molecular mass of the protein appeared to be 87 kDa. A similar procedure was used to characterize complex C, but the UV-crosslinking experiments failed to detect any proteins present in the complex C, probably due to the low abundance of the complex and low efficiency of the UV-crosslinking.

KEY RESEARCH ACCOMPLISHMENTS

- Identification of a novel Sp1 binding site that contributes to the maximal p53 basal transcriptional activity in breast cancer cells.
- Demonstration of the critical functional role of the newly identified regulatory element PE21 motif in OM-induced transcriptional suppression of the p53 gene.
- Providing the first evidence that an 87 kDa DNA binding protein interacts with the PE21 sequence.

REPORTABLE OUTCOMES

- One poster entitled "**Down-Regulation of p53 Tumor Suppressor Promoter Activity by Oncostatin M in Breast Cancer Cells**" was presented at the 92nd Annual Meeting of AACR
- One manuscript entitled "**The Critical Role of the PE21 Element in Oncostatin M-Mediated Transcriptional Repression of the p53 Tumor Suppressor Gene in Breast Cancer Cells**" was submitted to the journal Oncogene on June 11, 2001

CONCLUSIONS

Transcriptional regulation of the p53 gene contributes to the change in expression of wildtype p53 during the cell cycle and to the elevated expression of mutated p53 in tumor cells. However, currently, little is known regarding the regulation of p53 transcription in tumor cells. Characterization of the human p53 promoter to localize the OM responsive elements and to further identify the interacting transcription factors will provide new information for understanding the transcriptional control of p53 expression in breast cancer cells. We have now identified the PE21 motif as the OM-responsive element that mediates the inhibitory effect of OM on p53 transcription. We are currently working towards our Task II to examine the relationship between p53 expression and breast cancer cell differentiation.

Figure Legends

Figure 1. Schematic representation of p53 promoter luciferase reporter plasmid. A 599 bp fragment of the p53 gene covering –426 to + 172 was inserted into 5' *Kpn1* and 3' *BglII* sites of the promoter-less luciferase reporter pGL3-basic. The 5' deletion fragments of the p53 promoter were synthesized by PCR using pGL3-p53Luc as the template. The p53 promoter fragments were inserted into 5' *SacI* and 3' *XhoI* sites of pGL3-basic. The most 3' end of the major transcription initiation site for the human p53 gene is defined as +1 and the locations of the 5' ends of the promoters are indicated by the negative numbers of nucleotides relative to the transcription start site.

Figure 2. P53 promoter deletion and mutation analysis to define functional regulatory sequences that control the basal promoter activity of the p53 gene.
(A) Deletion analysis: p53Luc and 5' deletion constructs containing various lengths of the promoter were transfected into MCF-7 cells along with the vector pRL-TK. Cell lysate was harvested 40 h after transfection. The normalized luciferase activity of p53Luc is expressed as 100%. The data (mean ± SD) shown are derived from 6-8 separate transfection experiments in which triplicate wells were assayed.
(B) EMSA: A double stranded oligonucleotide corresponding to the p53 promoter region –183 to –154, designated as p53-Sp1, was radiolabeled and incubated with 10 μg of nuclear extract prepared from MCF-7 cell in the absence (lane 1) or in the presence of 100-fold molar unlabeled competitors (lane 2-3), or in the presence of antibodies (lane 4-6). The reaction mixture was loaded onto a 6% polyacrylamide gel and run in TGE buffer at 30 mA for 2.5 h at 4°C.
(C) Mutation analysis: p53Luc-wt and p53Luc-mSp1 were transiently transfected into MCF-7 along with the vector pRL-TK. The normalized luciferase activity of p53Luc-wt is expressed as 100%. The p53Luc-mSp1 exhibited 54.1% activity compared with p53Luc-wt.

Figure 3. OM down-regulates p53 promoter activity in a dose-dependent and a time-dependent manner. MCF-7 cells were transfected with p53Luc along with the vector pRL-TK. In A, after addition of DNA into the medium, OM dilution buffer or OM at different concentrations were added to the cells and cells were harvested 40 h later. In B, after addition of DNA into the medium, OM at a saturable concentration (50 ng/ml) was added to the cells at different times and cells were harvested together after 48 h of transfection. The normalized luciferase activity of transfected cells that were untreated is expressed as 100%. The data (mean ± S.D.) shown are representative of three independent transfection experiments in which triplicate wells were transfected for each condition.

Figure 4. The proximal region of the p53 promoter contains an OM-responsive element.

P53Luc and 5' deletion constructs containing various lengths of the promoter were transfected into MCF-7 cells along with the vector pRL-TK. After transfection, cells were incubated in the presence or absence of OM 50 ng/ml for 24 hours. The normalized luciferase activity of transfected cells that were untreated is expressed as 100%. The data (mean ± S.E.) shown were derived from 6-8 independent transfection

assays. Differences in normalized luciferase activities between untreated and OM treated samples were evaluated using two tailed Student's *t* test. A statistically significant difference ($p<0.05$) is indicated by an asterisk.

Figure 5. Localization of the OM response to the PE21 element in the human p53 promoter. P53 promoter reporter wild type and mutants were transfected into MCF-7 cells individually. After transfection, cells were incubated in the presence or absence of OM (50 ng/ml) for 40 hours. The data represent the results of 6-8 independent transfections. The normalized luciferase activity of each vector was expressed as the % of luciferase activity of p53Luc wild type vector in untreated control cells. An asterisk sign indicates that there is a statistically significant difference between the untreated control and the OM treated sample.

Figure 6. Effects of OM on heterologous promoter constructs containing the PE21 element. The pTKLuc-PE21 vectors were constructed by insertion of sequences of the PE21 element in tandem in sense (S) or antisense (As) orientation adjacent and upstream of the TATA box from the HSV tk promoter. The number indicates the number of repeat of the PE21 element in each construct. These vectors were transiently transfected into MCF-7 cells and examined for responses to OM treatment as described in Figure 5. The data shown are representative of 3-4 separate transfections.

Figure 7. EMSA analysis of nuclear proteins interacting with the PE21 motif. A double stranded oligonucleotide, designated as p53-PE21, was radiolabeled and incubated with 10 μg of nuclear extracts prepared from untreated (lanes 1-5) or OM 40 h treated MCF-7 cell (lanes 6-10) in the absence (lanes 1,6) or in the presence of 100-fold molar unlabeled competitors. The reaction mixture was loaded onto a 6% polyacrylamide gel and run in TGE buffer at 30 mA for 2.5 h at 4°C.

Figure 8. Denaturing SDS-polyacrylamide gel analysis of the UV cross-linked complex B formed with MCF-7 nuclear extract and the PE21 probe. The positions of ^{14}C-labeled molecular mass markers are shown in lane 1, and the protein detected from complex B is shown in lane 2. After correction for the bound oligonucleotide, the molecular mass of the band is 87 kDa. A very faint signal, possibly caused by insoluble materials, was seen in the interface of the stacking gel and the separating gel.

Table 1. Sequences of p53 promoter specific primers. Mutated nucleotides are in boldface and the binding sites are underlined.

Primer		Nucleotide sequence (5' to 3')
5' deletion PCR primers		
5'Del-2	5'	GAGCTCAAGCTTCTGCCCTCACAGCTCTGGCTTGCAG
5'Del-3		GAGCTCAAGCTTCACCCTCCTCCCCAACTCC
5'Del-4		GAGCTCAAGCTTGCTTTTGTGCCAGGAGCCTCG
5'Del-5		GAGCTCAAGCTTGCTCAAGACTGGCGCTAAAAGTT
	3'	GAAAATACGGAGCCGAGAGCC
EMSA oligonucleotides		
P53-Sp1	5'	GCACCCTCCTCCCCAACTCC
P53-mSp1	5'	GACTCTGCA**C**GCTC**G**TC**G**CCAACTCCATTTCCTTTGC
P53-PE21	5'	CCTCGCAGGGGTTGATGGGATTGGGGT
P53-mPE21	5'	CCTCGCAGGGGTTGATG**A**GC**T**C**G**GGGGT
Mutation primers		
P53Luc-mNFkB	5'	GGGGTTGATGGGATT**ATC**GTTTT**AAG**CTCCCATGTGC
P53Luc-m-c-myc	5'	GGGATTGGGGTTTTCCCCTCCC**T**TG**GA**CTCAAGACTGGC
P53Luc-mPE21	5'	GCCTCGCAGGGGTTGATG**A**GC**T**C**G**GGGGTTTTCCCCTCCC

REFERENCES

1. Zarling, J.M., Shoyab, M., Marquardt, H., Hanson, M.B., Lionbin, M.N., and Todaro, G.J. Oncostatin M: a growth regulator produced by differentiated lymphoma cells. Proc.Natl.Acad.Sci.USA., *83*: 9739-9743, 1986.

2. Horn, D., Fitzpatrick, W.C., Gompper, P.T., Ochs, V., Bolton-Hanson, M., Zarling, J.M., Malik, N., Todaro, G.J., and Linsley, P.S. Regulation of cell growth by recombinant oncostatin M. Growth Factors, *2*: 157-165, 1990.

3. Liu, J., Spence, M.J., Wallace, P.M., Forcier, K., Hellstrom, I., and Vestal, R.E. Oncostatin M-specific receptor mediates inhibition of breast cancer cell growth and down-regulation of the c-myc proto-oncogene. Cell Growth.&Differ., *8*: 667-676, 1997.

4. Spence, M.J., Vestal, R.E., and Liu, J. Oncostatin M-mediated transcriptional suppression of the c-myc gene in breast cancer cells. Cancer Research, *57*: 2223-2228, 1997.

5. Douglas, A.M., Goss, G.A., Sutherland, R.L., Hilton, D.J., Berndt, M.C., Nicola, N.A., and Begley, C.G. Expression and function of members of the cytokine receptor superfamily on breast cancer cells. Oncogene, *14*: 661-669, 1997.

6. Douglas, A.M., Grant, S.L., Goss, G.A., Clouston, D.R., Sutherland, R.L., and Begley, C.G. Oncostatin M induces the differentiation of breast cancer cells. Int.J.Cancer, *75*: 64-73, 1998.

7. Liu, J., Li, C., Ahlborn, T.E., Spence, M.J., Meng, L., and Boxer, L.M. The expression of p53 tumor suppressor gene in breast cancer cells is down-regulated by cytokine oncostatin M. Cell Growth.&Differ., *5*: 15-18, 1999.

8. Noda, A., Toma-Aiba, Y., and Fujiwaba, Y. A unique, short sequence determins p53 gene basal and UV-inducible expression in normal human cells. Oncogene, *19*: 21-31, 2000.

APPENDICES

I. Figures

Figure 1

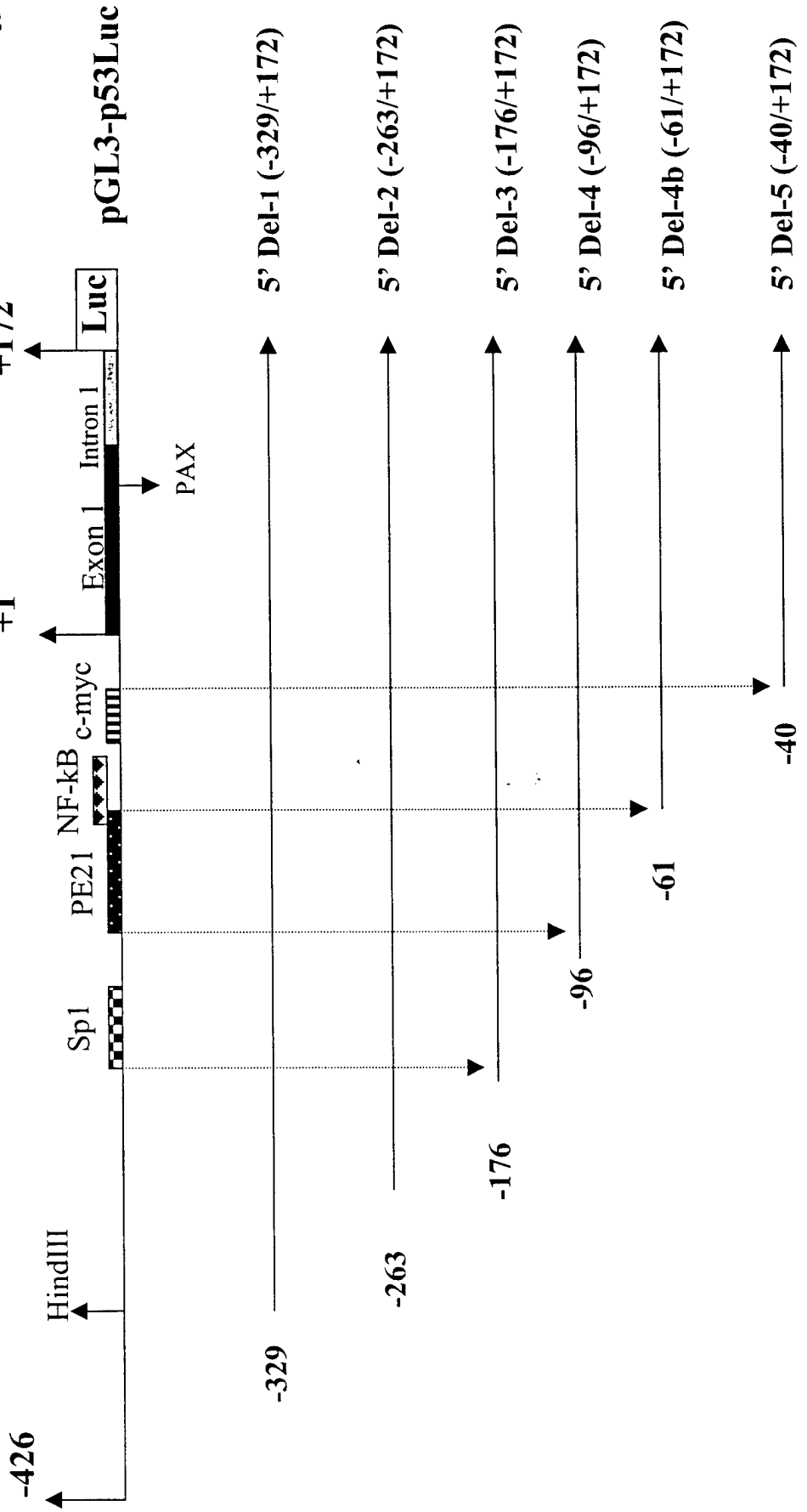
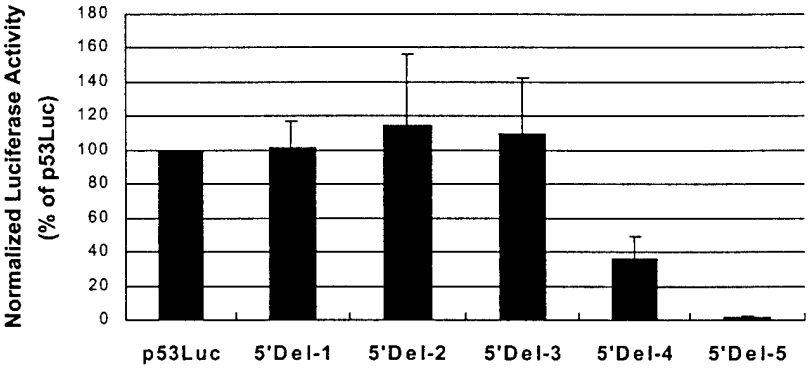
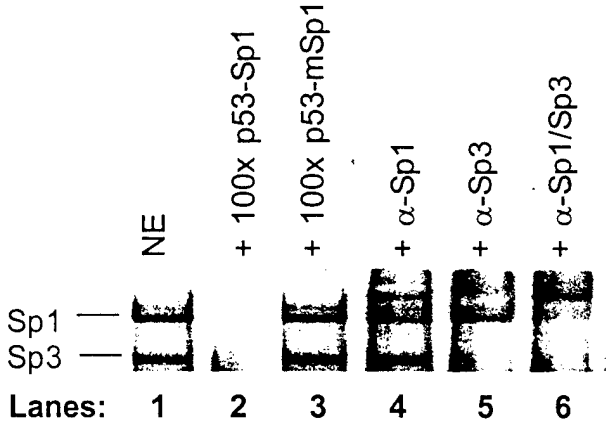


Figure 2

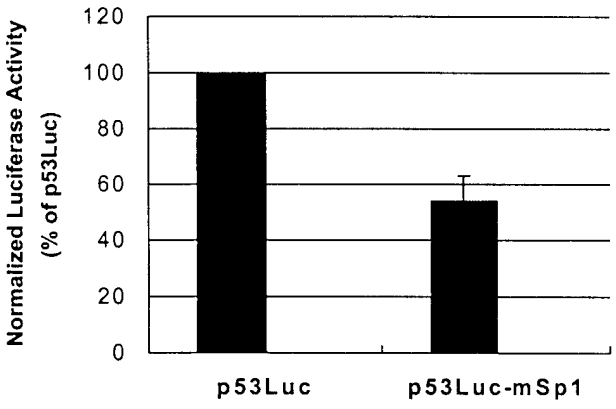
A


Normalized Luciferase Activity
(% of p53Luc)
180
160
140
120
100
80
60
40
20
0
p53Luc
5'Del-1
5'Del-2
5'Del-3
5'Del-4
5'Del-5

B


NE
+ 100x p53-Sp1
+ 100x p53-mSp1
+ α-Sp1
+ α-Sp3
+ α-Sp1/Sp3
Sp1
Sp3
Lanes: 1 2 3 4 5 6

C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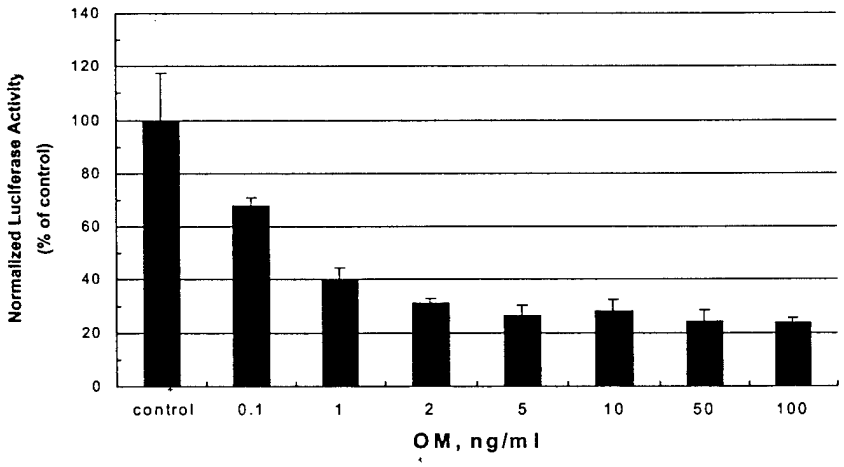
(% of p53Luc)
120
100
80
60
40
20
0
p53Luc
p53Luc-mSp1

Figure 3

A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(% of control)
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40
20
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control
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1
2
5
10
50
100
OM, ng/ml

B

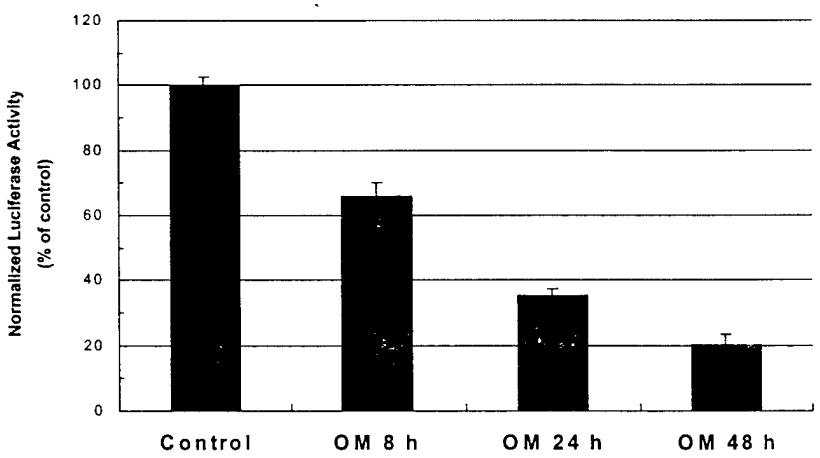

Normalized Luciferase Activity
(% of control)
120
100
80
60
40
20
0
Control
OM 8 h
OM 24 h
OM 48 h

Figure 4

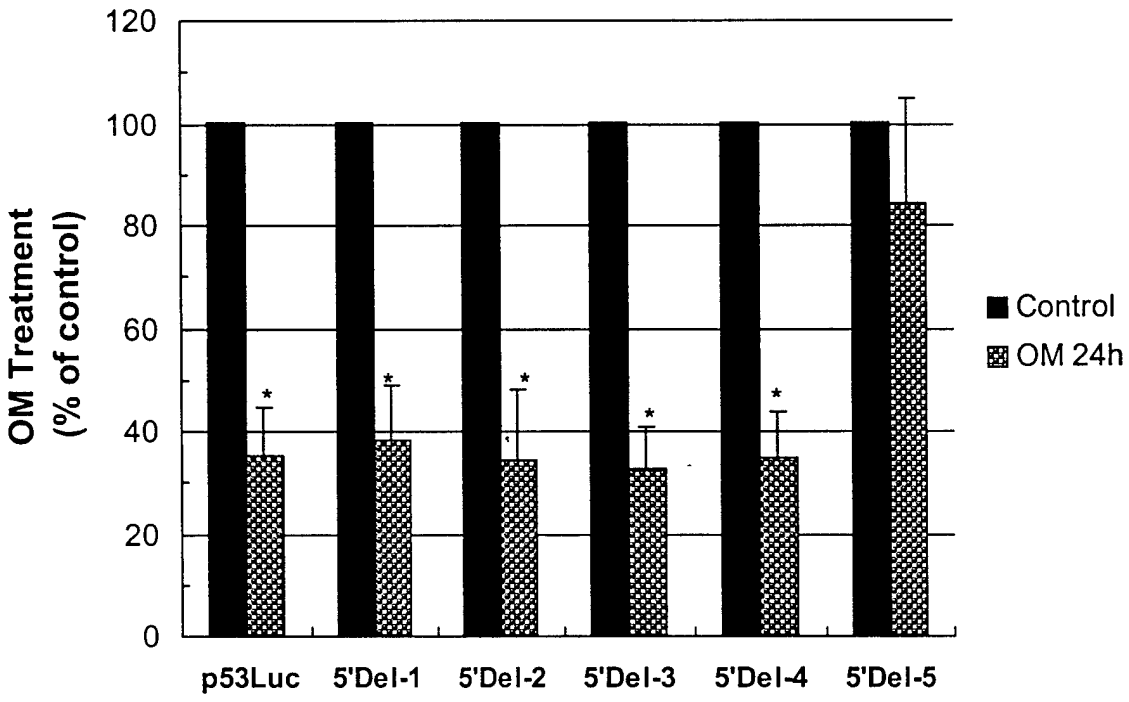

120
100
80
60
40
20
0
OM Treatment
(% of control)
p53Luc
5'Del-1
5'Del-2
5'Del-3
5'Del-4
5'Del-5
Control
OM 24h

Figure 5

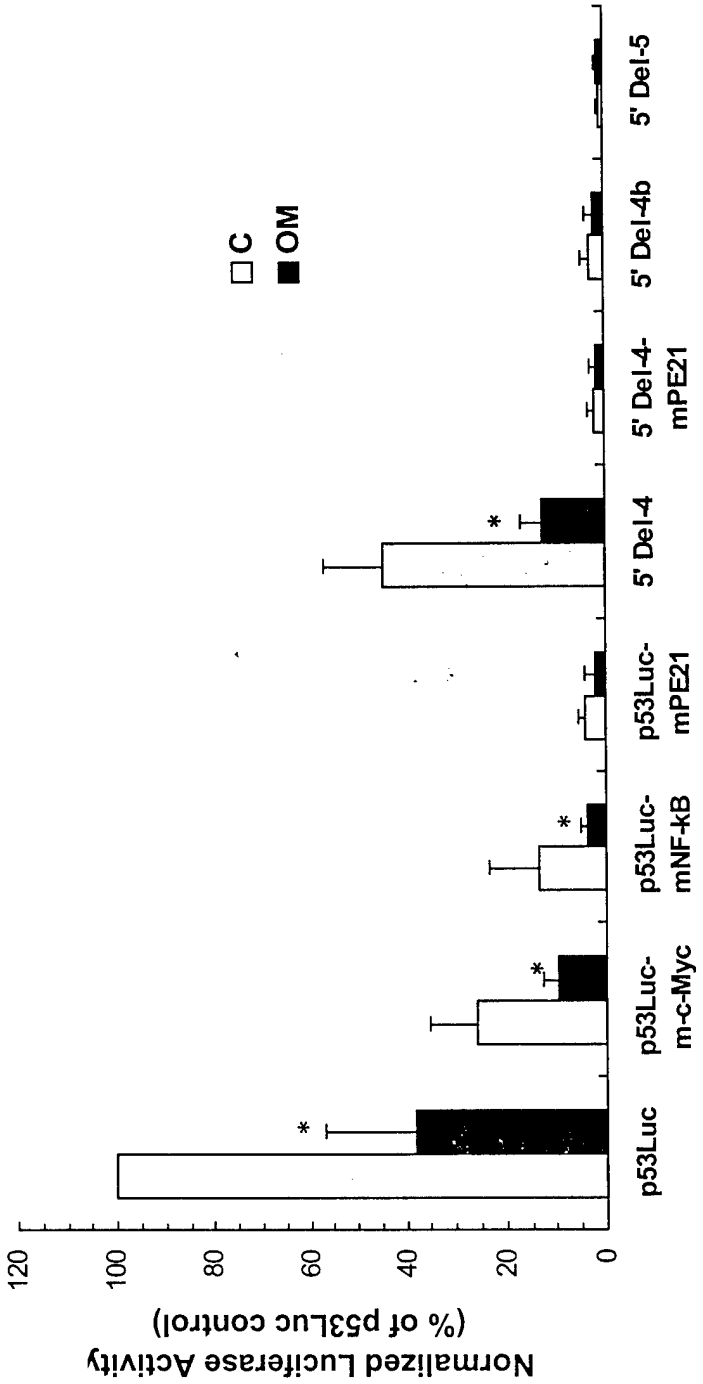

Normalized Luciferase Activity
(% of p53Luc control)
0
20
40
60
80
100
120
C
OM
*
p53Luc
p53Luc-m-c-Myc
p53Luc-mNF-kB
p53Luc-mPE21
5' Del-4
5' Del-4-mPE21
5' Del-4b
5' Del-5

Figure 6

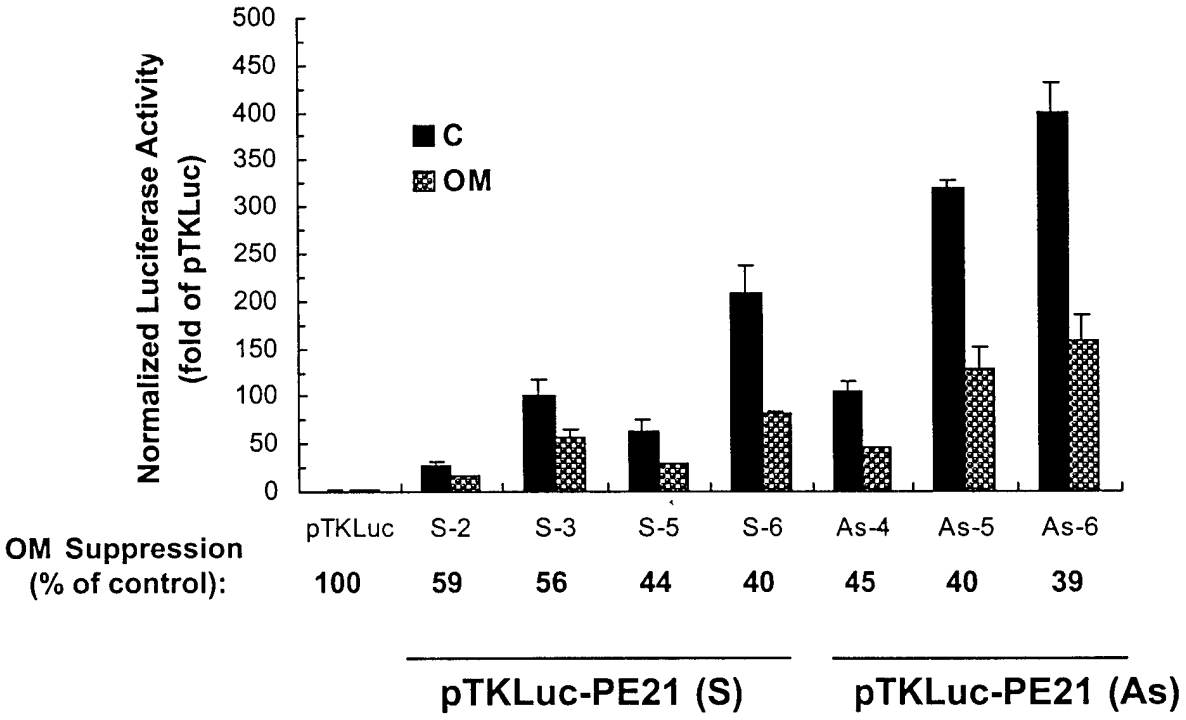

Normalized Luciferase Activity
(fold of pTKLuc)
500
450
400
350
300
250
200
150
100
50
0
C
OM
pTKLuc
S-2
S-3
S-5
S-6
As-4
As-5
As-6
OM Suppression
(% of control):
100
59
56
44
40
45
40
39
pTKLuc-PE21 (S)
pTKLuc-PE21 (As)

Figure 7

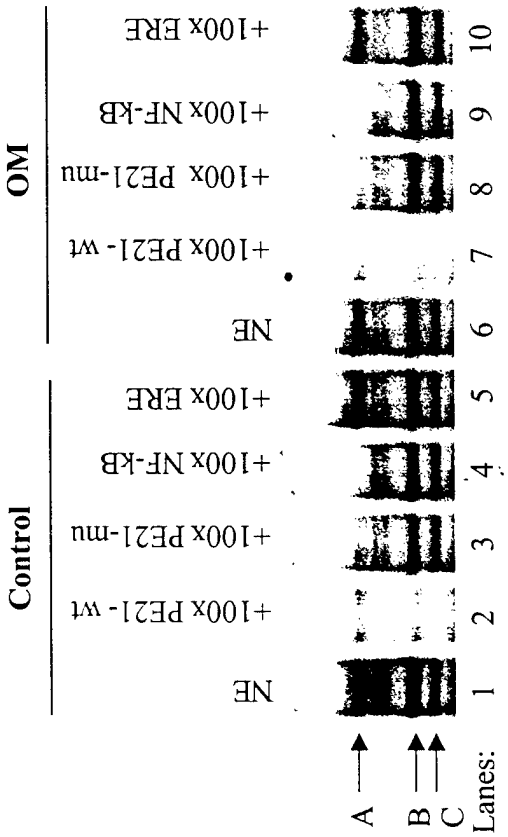
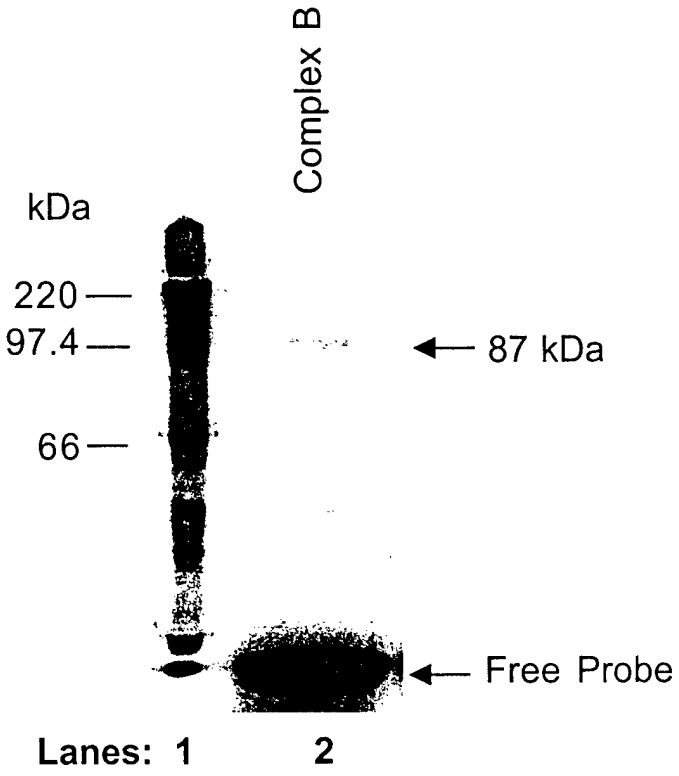

Control
OM
NE
+100x PE21- wt
+100x PE21-mu
+100x NF-kB
+100x ERE
NE
+100x PE21- wt
+100x PE21-mu
+100x NF-kB
+100x ERE
A
B
C
Lanes: 1 2 3 4 5 6 7 8 9 10

Figure 8



APPENDICES
II. AACR Poster Abstract

Down-Regulation of p53 Tumor Suppressor Promoter Activity by Oncostatin M in Breast Cancer Cells. C. Li, K. Tokita, T.E. Ahlborn, and J. Liu. *VA Palo Alto Health Care System, Palo Alto, CA 94304*

Compared to the vast information and knowledge available regarding the regulation of p53 protein expression, there is only a small amount of literature on transcriptional regulation of the p53 gene. Previously we have shown that p53 transcription is down-regulated by cytokine oncostatin M (OM) in growth inhibited and differentiated breast cancer cells. However the molecular mechanisms underlying the OM effect on p53 transcription is largely unknown. To characterize the cis-regulatory elements that are important for the basal transcriptional activity of p53 in breast cancer cells and to localize the promoter region that is responsible for OM-mediated suppression of p53, the wild-type p53 promoter (-420 to + 178) and a series of 5' deletion fragments of the p53 promoter were subcloned into luciferase reporter vector pGL3-basic and transiently transfected into MCF-7 cells. The results showed that deletion of 5' sequence up to –171, relative to the transcription start site, did not change the basal activity, deletion up to –91 lowered the basal activity to 35% of the full promoter, further deletion to –35 drastically reduced the promoter activity to less than 5% of the full promoter. These data demonstrate that the promoter region –90 to –35 that contains the NFkB and c-myc binding sites are critical in maintaining the basal transcription. However, the fact that deletion of sequence from –170 to –91 lost 65% of promoter activity suggest that this region may contain regulatory element that also contributes to the basal promoter activity. Using gel shift assays, we have identified a Sp1 binding site at –168 to –157. Disruption of Sp1 binding to this region lowered basal promoter activity 50%, thereby demonstrating that the Sp1 site at –168 to –157 is an important regulatory element for p53 transcription in breast cancer cells. Treatment of MCF-7 cells with OM induced a time-dependent suppression of p53 promoter activity. The p53 promoter activity was decreased to 35% of control at 24 h and further decreased to 20% at 48 h. By using 5' and 3' deletion constructs of p53 promoter we have localized the region –90 to –35 to be responsible for OM-mediated suppression. Experiments of mutagenesis and gel shift assays are currently being performed to identify the OM-responsive regulatory elements. Identification of cis-acting elements and trans-acting factors that are important for p53 gene transcription and regulation by 1oncostatin M in breast cancer cells will provide insight for understanding the functional roles of p53 in proliferation and differentiation of breast cancer cells.

APPENDICES

III. Manuscript submitted to Oncogene

The Critical Role of the PE21 Element in Oncostatin M-Mediated Transcriptional Repression of the p53 Tumor Suppressor Gene in Breast Cancer Cells

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Running Title

Repression of p53 gene transcription

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ABSTRACT

Cytokine oncostatin M (OM) exerts growth-inhibitory and differentiative effects on breast cancer cells. Previously we showed that the transcription of the p53 gene in breast cancer cells was down regulated by OM. To elucidate the molecular mechanisms underlying the OM effect on p53 transcription, in this report, we dissected the p53 promoter region and analyzed the p53 promoter activity in breast tumor cells. We showed that treatment of MCF-7 cells with OM induced a dose- and time-dependent suppression of p53 promoter activity. The p53 promoter activity was decreased to 35% of control at 24 h and further decreased to 20% at 48 h by OM at concentrations of 5 ng/ml and higher. Deletion of the 5'-flanking region of the p53 promoter from –426 to –97 did not affect the OM effect. However, further deletion to –40 completely abolished the repressive effect of OM. The p53 promoter region –96 to –41 contains NF-kB and c-myc binding sites, and a newly identified UV-inducible element PE21. Mutations to disrupt NF-kB binding or c-myc binding to the p53 promoter decreased the basal promoter activity without affecting the OM-mediated suppression, whereas mutation at the PE21 motif totally abolished the OM effect. We further demonstrated that insertion of PE21 element upstream of the thymidine kinase minimal promoter generated an OM response analogous to that of the p53 promoter. Finally, we detected the specific binding of a nuclear protein with a molecular mass of 87 kDa to the PE21 motif. Taken together, we demonstrate that OM inhibits the transcription of the p53 gene through the PE21 element. Thus, the PE21 element is functionally involved in p53 transcription regulated by UV-induction and OM suppression.

INTRODUCTION

The p53 tumor suppressor protein is involved in several central cellular processes that are critical for maintaining cellular homeostasis, including gene transcription (1; 2), DNA repair (3; 4), cell cycling (5; 6), senescence (7; 8), and apoptosis (9; 10). Compared to the vast information and knowledge available regarding the regulation of p53 protein expression and function (11-13), there is only a small amount of literature on transcriptional regulation of the p53 gene (14-20). However, control of p53 gene expression at the transcriptional level has been shown to play important roles in mitogenic stimulation or factor induced differentiation (21-23). Moreover, the deregulated transcription of p53 accounts for at least in part, the elevated expression of mutant p53 in tumor cells (23).

Since cloning of the human p53 promoter in 1989 (Tuck *et al.*, 1989), several transcription factors have been identified that interact with specific regions of the p53 promoter to positively or negatively regulate transcription. The transcription factors shown to positively regulate p53 transcription include c-myc (15; 20), NF-kB (16; 20; 24), YY1/NF1 (17-19; 25), Ap1 (20), and the HoxA5 homeobox containing gene product (26). Members of the PAX family are the only mammalian nuclear proteins shown to repress p53 transcription through a binding site present in the first non-coding exon (Stuart *et al.*, 1995).

Recently, a novel 21 bp motif, named the PE21 element, was identified in the human p53 promoter that is located immediately upsteam of the NF-kB binding site (27). It was shown that the PE21 element covering the region of –79 to –59 is a primary determinant for the basal transcription of the p53 gene and the sequence required for UV-induced transcription in human fibroblasts. Mutations within this region drastically reduced the basal promoter activity and abolished the UV-induction. Interestingly, this 21 bp motif appears to have a function in initiation of transcription in a bi-directional manner. Insertion of multiple copies of PE21 in the sense or antisense orientiation into a promoterless luciferase reporter pGL2-basic initiated the transcription of the luciferase gene and generated an UV-inducible response as well. It remains to be elucidated whether the PE21 element has a functional role in p53 transcription regulated by cellular factors or other extracellular stimuli. Furthermore, the PE21 binding proteins need to be identified and characterized.

Oncostatin M (OM), a 28 kDa glycoprotein, is a cytokine produced by activated T lymphocytes and macrophages (28). Previous studies showed that OM inhibits the growth of several breast cancer cell lines, including MCF-7, MDA-MB231, and H3922, which is a cell line derived from an infiltrating ductal carcinoma (29-33). Breast cancer cells respond to OM treatment with reduced growth rates and the appearance of differentiated phenotypes. However, OM treatment does not appear to lead to apoptosis. Since the p53 tumor suppressor protein plays important roles in cellular proliferation and differentiation, we examined the effects of OM on p53 expression in breast cancer cells. Surprisingly, we found that p53 expression was down regulated by OM in MCF-7, MDA-MB231, and H3922 cells (34). Decreased levels of p53 protein and mRNA were detected after 1 day of OM treatment and reached maximal suppression of 10-20% of control after 3 days. Nuclear run-on assays further demonstrated that OM decreased the number of actively transcribed p53 mRNA. These studies suggest that OM may repress p53 gene transcription. The effect of OM on p53 transcription appears to precede its effects on cell

growth inhibition and induction of morphological changes, as the retardation of cell growth by OM could be only detected after 3-4 days but a decrease in the level of p53 mRNA could be detected as early as 6-8 hours (34).

In order to delineate the molecular mechanisms by which OM regulates p53 transcription and to understand the relationship between p53 expression and proliferation and differentiation of breast cancer cells, in this study we dissected the p53 promoter region to identify the cis-acting element that mediates the OM effect in MCF-7 cells. Our results demonstrate that the effect of OM is not mediated through the known repressor PAX binding site. Instead the PE21 element is responsible for OM-induced suppression of p53 transcription. Mutation of PE21 in the context of the p53 promoter completely abolished the inhibitory activity of OM on p53 transcription. By contrast, insertion of the PE21 motif into an OM-unresponsive TK promoter created a phenomenon of transcriptional suppression, resembling the p53 promoter.

RESULT

Deletion analysis to define the regulatory sequences involved in the basal transcriptional activity of the p53 promoter in breast tumor cells

The regulatory sequences that control p53 transcription in breast tumor cells have not been clearly defined, although a number of studies had examined p53 promoter activity in other cell types. Thus, initially in the present study, we generated a series of reporter constructs in which luciferase gene is driven by varying lengths of the 5'-flanking region of the p53 gene. These constructs were tested for activity in MCF-7 cells. A diagram of the deletion constructs is shown in Figure 1. Figure 2A compared the basal promoter activity of the deletion constructs with the activity of the full promoter construct pGL3-p53Luc that contains a 599 bp fragment of the p53 promoter (-426 to +172) (34). These results, representing 6-8 separate transfections, showed that deletion of the 5'-flanking region from –426 to –177 did not affect the p53 promoter activity, whereas deletion down to –97 (5' Del-4) significantly lowered the basal activity to approximately 40% of the full promoter. Further deletion to –41 (5' Del-5) to eliminate the binding sites for NF-kB and c-myc drastically reduced the basal promoter activity to a level below 5% of the full promoter. These data suggest that the transcription factors NF-kB and c-myc play critical roles in the basal transcriptional activity of the p53 gene in breast tumor cells, however, the promoter region covering –176 to –97 may contain a regulatory sequence that is responsible for the maximal basal transcriptional activity of the p53 gene in MCF-7 cells.

The p53 promoter region from –176 to –97 contains a stretch of CT rich sequence (CCCTCCTCCCC -174 to –164), a potential binding site for the transcription factor Sp1. To determine whether Sp1 interacts with this sequence, electrophoretic mobility shift assay (EMSA) was conducted with the nuclear extract isolated from MCF-7 cells and a ^{32}P labeled double-stranded oligonucleotide, p53-Sp1, corresponding to the promoter region –183 to –154. Upon incubation of p53-Sp1 with nuclear extract, two DNA-protein complexes were detected (Figure 2B, lane 1). Formation of these complexes was inhibited by competition with a 100-fold molar excess of the unlabeled probe p53-Sp1 (lane 2), but was not inhibited by the oligonucleotide p53-mSp1 that contains mutations within the CT-stretch (lane 3). The faster moving complex was supershifted by the anti-Sp3 antibody (lane 5), whereas the slower moving complex was partially supershifted by

the anti-Sp1 antibody (lane 4). Inclusion of anti-Sp1 and anti-Sp3 antibodies together in the reaction mixture completely supershifted both complexes (lane 6). These data demonstrate that transcription factors Sp1 and Sp3 bind to this CT-rich region of the p53 promoter.

To determine the function of Sp1/Sp3 in mediating the p53 basal promoter activity, the Sp1 site in p53Luc was mutated (CCCTCCTCCCC to CGCTCGTCGCC) and the mutated reporter p53Luc-mSp1 along with the wild type vector p53Luc were transfected into MCF-7 cells. Figure 2C show that mutation of this Sp1 site lowered the p53 promoter activity by approximately 50%, thereby suggesting that loss of the Sp1/Sp3 binding to the CT-rich region is primarily responsible for the diminished basal promoter activity of the deletion mutant 5' Del-4. These results together demonstrate that Sp1 and Sp3 are positive trans-activators of p53 transcription and that their binding to the CT rich sequence contributes to the basal transcriptional activity of the p53 gene.

Dose-dependent and time-dependent responses of p53 transcription to OM

Next, the effect of OM on p53 promoter activity in MCF-7 cells was examined. The full promoter construct p53Luc was transiently transfected into MCF-7 cells along with the renilla luciferase expression vector pRL-TK. After transfection, cells were untreated or treated with OM at different concentrations for 48 h. Figure 3A shows that the suppressive effect of OM on p53 promoter activity was detected at 0.1 ng/ml, and a maximal suppression of 75-80% of p53 promoter activity was observed at 5 ng/ml. The inhibitory effect of OM on p53 transcription was also time-dependent. The p53 promoter activity was decreased to 67% of control by 8 h, lowered to 35% by 24 h, and further declined to 20% of control by 48 h after treating cells with a saturable concentration of OM. These results clearly demonstrate that OM represses p53 promoter activity in a dose-dependent and a time-dependent manner that is directly correlated with the effects of OM on p53 mRNA expression, as we previously reported (34).

Dissection of the p53 promoter to define the OM-responsive region

To define the OM-responsive region in the p53 promoter, the 5' and 3' deletion constructs of the p53 promoter were transfected into MCF-7 cells. Then the transfected cells were untreated or treated with OM for 40 h prior to cell lysis. The results of 6-8 transfection assays using 5' deletion constructs are summarized in Figure 4. These results showed that deletion of the 5'-flanking region from –426 to –97 did not affect the OM response. In contrast, further deletion to –41 (5' Del-5) eliminated the OM effect. These data suggest that the promoter region covering –96 to –40 is not only important for the basal transcriptional activity as shown in Figure 2A but it may also contain the critical OM-responsive element. Furthermore, shortening of the 3' region from +172 to +14 to delete the PAX binding site had no effect on OM-mediated suppression or the basal promoter activity, thereby excluding the involvement of the repressor PAX in OM-mediated down regulation of p53 transcription (data not shown). ***Localization of the OM-responsive sequence to the PE21 element***

The 5' deletion analysis localized the OM-responsive sequence to the proximal region of the p53 promoter from –96 to –41. This region contains three important regulatory motifs including NF-kB, the E-box (c-myc), and the newly identified UV-inducible PE21 element. To investigate the role of these regulatory sequences in OM-mediated suppression, site-directed mutagenesis was conducted on the full promoter p53Luc to mutate each binding site individually. Figure 5 shows that mutation of the c-

myc site lowered the basal promoter activity 75% without affecting the OM effect. Likewise, mutation of the NF-kB binding site decreased the basal promoter activity 85% with little effect on OM. By contrast, mutation at the PE21 element drastically reduced the basal promoter activity and rendered the p53 promoter unresponsive to OM. To confirm this finding, the PE21 element was mutated in the vector 5'Del-4 that contains the minimal sequence for the basal transcription and the OM-mediated suppression. Again, the OM inhibitory effect was not observed in the PE21 mutant in the context of this short promoter fragment. Similarly, deletion of the PE21 motif from the 5' Del-4 (5' Del-4b) resulted in the loss of the OM response.

Next, we were interested in determining whether OM could exert its effect on PE21 in the context of a heterologous promoter that contains the PE21 element without auxiliary sequences of p53 promoter. To test this, luciferase reporters containing different copies of PE21 in tandem inserted 5' upstream of a minimal HSV tk promoter (pTKLuc) in either sense, pTKLuc-PE21 (S), or antisense, pTKLuc-PE21 (As) orientations were transfected into MCF-7 cells. The plasmid pTKLuc produced low but measurable luciferase activity in MCF-7 cells and OM treatment did not lower the activity. Inclusion of the PE21 sequence in either direction greatly increased luciferase activities from 20-fold to 400-fold of the pTKLuc. The fold increase of luciferase activity was concurrent with the increase in PE21 copy number in most cases and showed a preference with the antisense orientation. Importantly, in contrast to the vector pTKLuc, the reporters containing the PE21 element clearly displayed responses to OM with luciferase activities reduced to 39% to 59% of control in the OM treated cells, comparable to the OM effect observed in the native p53 promoter. Together, our results presented in Figures 5 and 6 provide strong evidence that the PE21 element plays an important role in the basal transcription of the p53 gene and is also critically involved in the OM-induced transcriptional suppression of the p53 gene in breast cancer cells.

Characterization of nuclear proteins that interact with the PE21 element

To detect nuclear proteins in MCF-7 cells that specifically interact with the PE21 sequence, EMSA was conducted with ^{32}P-labeled oligonucleotide p53-PE21 containing the PE21 and flanking sequence, and nuclear extracts prepared from untreated or OM-treated cells. Figure 7 shows that 3 specific complexes were detected in both control and OM-treated nuclear extracts. The formation of these complexes was inhibited with 100x-fold molar excess of unlabeled probe (lanes 2, 7) but was not inhibited by a 100x-fold molar excess of an unrelated DNA containing an estrogen response element (lanes 5, 10). An oligonucleotide containing the NF-kB site of the p53 promoter competed for the binding of complex A but not complexes B and C (lanes 4, 9). The binding of complex A was also competed by oligonucleotide p53-mPE21 that contains a 3 bp mutation within the PE21 sequence (lanes 3, 8). Since the PE21 probe covers the positions –82 to –56 including the 5' end of the NF-kB site, we believe that complex A contains subunits of nuclear factor NF-kB.

Complexes B and C are PE21 specific as the oligonucleotide p53-mPE21 lost the ability to compete with the binding of these two complexes to the labeled PE21 probe. Apparently OM treatment did not altered the pattern of the complexes or the intensity of the binding signals. This observation was consistent with the results of UV-induction. It was shown that the binding of nuclear proteins of fibroblasts without or with UV-irradiation to the PE21 probe was not different (27).

Previous studies conducted in human fibroblasts did not characterize the protein/DNA complex of the PE21 sequence. It is unknown whether a single DNA binding protein or multiple proteins interact with the PE21 motif. To characterize the MCF-7 nuclear proteins present in the PE21 DNA complexes, EMSA was followed by UV cross-linking. Complex B was excised from the gel and the protein components were analyzed by denaturing SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Analysis of the SDS-PAGE revealed that one protein was crosslinked to the labeled PE21 probe (Figure 8). After correction for the bound oligonucleotide, the molecular mass of the protein appeared to be 87 kDa. A similar procedure was used to characterize complex C, but the UV-crosslinking experiments failed to detect any proteins present in the complex C, probably due to the low abundance of the complex and low efficiency of the UV-crosslinking.

DISCUSSION

The PE21 element was originally discovered by searching for the sequence that was responsible for the UV-induced transcription of the p53 gene in human fibroblasts (27). Intriguingly, in this study, we found that the repressive effect of OM on p53 transcription was also mediated through this regulatory element. Our studies further highlight the importance of the PE21 element in the control of p53 gene transcription.

Our studies clearly demonstrate that the PE21 element is a critical sequence that controls p53 transcription in breast cancer cells, as mutation of this sequence produced the severest impact on p53 promoter activity in MCF-7 cells as compared to mutations on other functional sites such as NF-kB or the bHLH c-myc binding site. Mutations to disrupt the binding of NF-kB to its recognition sequence adjacent to PE21 lowered the basal promoter activity to 15% of the wild type promoter; mutation of the basic HLH site to interfere the binding of c-myc reduced the basal transcription by 75%. In contrast, alteration of 3 bp within the PE21 motif nearly eliminated the transcriptional activity of the p53 promoter. The promoter activity with the PE21 mutant was less than 5% of the wild type promoter. The ability of PE21 in activation of gene transcription was further demonstrated by its strong inducing effect on pTKLuc that contains a weak promoter composed a TATA box and one non-functional Sp1 site.

In this study we showed that mutations of the PE21 element in the context of the full p53 promoter (p53Luc) or in the context of the short promoter fragment (5' Del-4) that retains 40% of the p53 promoter activity completely eliminated the inhibitory effect of OM on p53 transcription. In contrast, mutations at other functional sites including NF-kB or c-myc did not abolish the OM inhibitory activity. The critical role of the PE21 site in OM-mediated repression of p53 transcription is further supported by the results of transfection with the pTKLuc-PE21 reporters. OM had no effect on the promoter activity of pTKLuc. However, the promoter activities of pTKLuc-PE21 in either the sense or antisense orientation were clearly suppressed by OM to levels comparable to that observed in the p53 promoter. These results suggest that the PE21 element is the primary cis-acting sequence that mediates the OM-induced transcriptional repression of the p53 gene.

We were able to detect two DNA-protein complexes formed with the PE21 sequence from MCF-7 cells. Complex B was relatively more abundant than complex C. EMSA experiments followed by UV-cross linking and SDS-PAGE revealed that a

nuclear protein with a molecular mass around 87 kDa was present in complex B. The relationship between complex B and complex C is presently unknown. It is possible that more than one nuclear protein binds to the PE21 sequence. Alternatively, the faster moving complex C may represent a degradation product of the protein in complex B.

OM treatment did not alter the binding of nuclear proteins to PE21; the same two complexes were detected in the OM-treated sample as in the control. This result is not totally surprising as the previous studies with UV-induction did not detect a different binding pattern with the PE21 sequence and nuclear extracts isolated from UV-irradiated and non irradiated fibroblasts (27). Our results combined with the prior study suggest that regulation of p53 transcription through the PE21 element by OM is mediated by mechanisms other than direct alteration of the DNA binding activity of the PE21 interacting proteins. It is possible that there are other cofactors associated with the PE21 binding protein. OM treatment could interfere with this association. The inability to detect changes of DNA binding activities of transcription factors to a functional regulatory element by gel shift assays has been described in other promoter studies. For example, the transcription of $p21^{WAF1/CIP1}$ gene is activated by TGFβ, phobol esters, and histone deacetylase inhibitors through a Sp1 site proximal to the p21 promoter. However, none of these activators altered the DNA binding activity of Sp1 (35-39).

Previous studies have defined the p53 promoter as a TATA-less and GC-rich less promoter, as the TATA-box and GC-rich sequence are not present in the proximal promoter region of the p53 gene (Tuck *et al.*, 1989). However, our studies with deletion and mutation analysis identified a novel Sp1/Sp3 binding site that covers the region –174 to –165 of the p53 promoter. This Sp1 binding site is expendable for OM-mediated suppression of p53 transcription but is important for the basal transcriptional activity of p53 gene. Mutation or deletion of this CT-rich sequence decreased p53 promoter activity by 50-60%. The involvement of Sp1/Sp3 in p53 transcription is further demonstrated by our EMSA supershift assay that clearly showed Sp1 and Sp3 binding to this region. Therefore, it is highly likely that Sp1 as a ubiquitously expressed transcription factor has a functional role in p53 gene transcription.

In summary, our studies demonstrate that p53 transcription is down regulated by OM in growth-inhibited and differentiated breast cancer cells. The PE21 element mediates this repressive effect. It is interesting to speculate that the activities of several different intracellular signal transduction pathways converge at the PE21 element. The UV-induced activation of p53 transcription is linked to a stress signal. The OM-induced suppression likely involves the MAP kinase ERK pathway, as we have found that the effects of OM on p53 protein expression and on p53 promoter activity can be partially blocked by the MEK inhibitor PD98059 (34). Further studies to identify and characterize the PE21 element binding proteins will greatly facilitate the understanding of the role of the PE21 site in the control of p53 transcription and its connection to intracellular signaling in normal cells as well as in tumor cells.

MATERIALS AND METHODS

Cells and reagent- The human breast cancer cell line MCF-7 was obtained from American Type Culture Collection (Manassas, VA) and cultured in RPMI-1640 medium supplemented with 10% heat inactivated fetal bovine serum (FBS).

P53 promoter luciferase reporters-pGL3-p53Luc contains a 599 bp fragment of the human p53 promoter region and exon 1 (-426 to + 172) (Liu et al., 1999). To construct 5'del-1 (-329 to +172), the pGL3-p53Luc was digested with restriction enzyme *HindIII*. The DNA fragment was isolated and subcloned into the *HindIII* site of pGL3-basic vector. Additional 5' and 3' deletion constructs were made by PCR with pGL3-p53Luc as the template. Table I describe the primer sequence of oligonucleotides utilized in the deletion and mutation analysis, as well as in EMSAs.

Transient transfection and luciferase assay – MCF-7 cells were plated at a density of 80,000 cells/well in 24 well plates and incubated for 24 h. Cells were cotransfected with 90 ng of various p53 promoter reporter plasmids and 10 ng of a pRL-TK as a normalizing vector per well by using Effectene transfection reagent (Qiagen). Transfected cells were incubated with human recombinant OM at a saturable concentration of 50 ng/ml or the OM dilution buffer (BSA 1 mg/ml in PBS) for 40 h prior to cell lysis. Luciferase activities were measured using the Promega Dual Luciferase Assay System. All the measured fire fly luciferase activity of the plasmid constructs was divided by the renilla luciferase activity of pRL-TK to normalize the transfection efficiency.

Electrophoretic mobility shift assays (EMSA)- Nuclear extracts of MCF-7 cells were prepared as previously described (40). Ten micrograms of nuclear extract were incubated with a ^{32}P-labeled 27 bp oligonucleotide containing the PE21 sequence for 15 min at RT and loaded onto 6% polyacrylamide gels and run in TGE buffer at 30 mA for 2.5 h at 4°C. The gels were dried and visualized on a PhosphorImager. For UV-cross-linking, the wet gel was briefly exposed to a PhosphoImager screen to locate the complexes. The region of the gel containing complex B was cut out, and the proteins were eluted at room temperature overnight in elution buffer containing 50 mM Tris-HCL (pH 7.9), 0.1% SDS, 0.1 mM EDTA, 5 mM DTT, 150 mM NaCl, and 50 μg/ml gamma-globulin. The eluted protein was precipitated with 4 volumes of dry ice-cold acetone, washed with ethanol, and air-dried. After resuspension in Laemmili loading buffer and heating, SDS-polyacrylamide gel electrophoresis was performed and the labeled protein was visualized by a PhosphorImager.

Footnotes

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3. The abbreviations used are:

EMSA	=	electrophoretic mobility shift assay
FBS	=	fetal bovine serum
OM	=	oncostatin M
TK	=	thymidine kinase

Table 1. Sequences of p53 promoter specific primers. Mutated nucleotides are in boldface and the binding sites are underlined.

Primer		Nucleotide sequence (5' to 3')
5' deletion PCR primers		
5'Del-2	5'	GAGCTCAAGCTTCTGCCCTCACAGCTCTGGCTTGCAG
5'Del-3		GAGCTCAAGCTTCACCCTCCTCCCCAACTCC
5'Del-4		GAGCTCAAGCTTGCTTTTGTGCCAGGAGCCTCG
5'Del-5		GAGCTCAAGCTTGCTCAAGACTGGCGCTAAAAGTT
	3'	GAAAATACGGAGCCGAGAGCC
EMSA oligonucleotides		
P53-Sp1	5'	GCACCCTCCTCCCCAACTCC
P53-mSp1	5'	GACTCTGCAC**G**CTC**G**TC**G**CCAACTCCATTTCCTTTGC
P53-PE21	5'	CCTCGCAGGGGTTGATGGGATTGGGGT
P53-mPE21	5'	CCTCGCAGGGGTTGATG**A**GCTC**G**GGGGT
Mutation primers		
P53Luc-mNFkB	5'	GGGGTTGATGGGATT**ATC**GTTTT**AAG**CTCCCATGTGC
P53Luc-m-c-myc	5'	GGGATTGGGGTTTTCCCCTCCC**T**TG**GA**CTCAAGACTGGC
P53Luc-mPE21	5'	GCCTCGCAGGGGTTGATG**A**GCTC**G**GGGGTTTTCCCCTCCC

Figure 1. Schematic representation of p53 promoter luciferase reporter plasmid. A 599 bp fragment of the p53 gene covering –426 to + 172 was inserted into 5' *Kpn1* and 3' *BglII* sites of the promoter-less luciferase reporter pGL3-basic. The 5' deletion fragments of the p53 promoter were synthesized by PCR using pGL3-p53Luc as the template. The p53 promoter fragments were inserted into 5' *SacI* and 3' *XhoI* sites of pGL3-basic. The most 3' end of the major transcription initiation site for the human p53 gene is defined as +1 and the locations of the 5' ends of the promoters are indicated by the negative numbers of nucleotides relative to the transcription start site.

Figure 2. P53 promoter deletion and mutation analysis to define functional regulatory sequences that control the basal promoter activity of the p53 gene.
(A) Deletion analysis: p53Luc and 5' deletion constructs containing various lengths of the promoter were transfected into MCF-7 cells along with the vector pRL-TK. Cell lysate was harvested 40 h after transfection. The normalized luciferase activity of p53Luc is expressed as 100%. The data (mean ± SD) shown are derived from 6-8 separate transfection experiments in which triplicate wells were assayed.
(B) EMSA: A double stranded oligonucleotide corresponding to the p53 promoter region –183 to –154, designated as p53-Sp1, was radiolabeled and incubated with 10 μg of nuclear extract prepared from MCF-7 cell in the absence (lane 1) or in the presence of 100-fold molar unlabeled competitors (lane 2-3), or in the presence of antibodies (lane 4-6). The reaction mixture was loaded onto a 6% polyacrylamide gel and run in TGE buffer at 30 mA for 2.5 h at 4°C.
(C) Mutation analysis: p53Luc-wt and p53Luc-mSp1 were transiently transfected into MCF-7 along with the vector pRL-TK. The normalized luciferase activity of p53Luc-wt is expressed as 100%. The p53Luc-mSp1 exhibited 54.1% activity compared with p53Luc-wt.

Figure 3. OM down-regulates p53 promoter activity in a dose-dependent and a time-dependent manner. MCF-7 cells were transfected with p53Luc along with the vector pRL-TK. In A, after addition of DNA into the medium, OM dilution buffer or OM at different concentrations were added to the cells and cells were harvested 40 h later. In B, after addition of DNA into the medium, OM at a saturable concentration (50 ng/ml) was added to the cells at different times and cells were harvested together after 48 h of transfection. The normalized luciferase activity of transfected cells that were untreated is expressed as 100%. The data (mean ± S.D.) shown are representative of three independent transfection experiments in which triplicate wells were transfected for each condition.

Figure 4. The proximal region of the p53 promoter contains an OM-responsive element.

P53Luc and 5' deletion constructs containing various lengths of the promoter were transfected into MCF-7 cells along with the vector pRL-TK. After transfection, cells were incubated in the presence or absence of OM 50 ng/ml for 24 hours. The normalized luciferase activity of transfected cells that were untreated is expressed as 100%. The data (mean ± S.E.) shown were derived from 6-8 independent transfection assays. Differences in normalized luciferase activities between untreated and OM treated

samples were evaluated using two tailed Student's *t* test. A statistically significant difference ($p<0.05$) is indicated by an asterisk.

Figure 5. Localization of the OM response to the PE21 element in the human p53 promoter. P53 promoter reporter wild type and mutants were transfected into MCF-7 cells individually. After transfection, cells were incubated in the presence or absence of OM (50 ng/ml) for 40 hours. The data represent the results of 6-8 independent transfections. The normalized luciferase activity of each vector was expressed as the % of luciferase activity of p53Luc wild type vector in untreated control cells. An asterisk sign indicates that there is a statistically significant difference between the untreated control and the OM treated sample.

Figure 6. Effects of OM on heterologous promoter constructs containing the PE21 element. The pTKLuc-PE21 vectors were constructed by insertion of sequences of the PE21 element in tandem in sense (S) or antisense (As) orientation adjacent and upstream of the TATA box from the HSV tk promoter. The number indicates the number of repeat of the PE21 element in each construct. These vectors were transiently transfected into MCF-7 cells and examined for responses to OM treatment as described in Figure 5. The data shown are representative of 3-4 separate transfections.

Figure 7. EMSA analysis of nuclear proteins interacting with the PE21 motif. A double stranded oligonucleotide, designated as p53-PE21, was radiolabeled and incubated with 10 μg of nuclear extracts prepared from untreated (lanes 1-5) or OM 40 h treated MCF-7 cell (lanes 6-10) in the absence (lanes 1,6) or in the presence of 100-fold molar unlabeled competitors. The reaction mixture was loaded onto a 6% polyacrylamide gel and run in TGE buffer at 30 mA for 2.5 h at 4°C.

Figure 8. Denaturing SDS-polyacrylamide gel analysis of the UV cross-linked complex B formed with MCF-7 nuclear extract and the PE21 probe. The positions of ^{14}C-labeled molecular mass markers are shown in lane 1, and the protein detected from complex B is shown in lane 2. After correction for the bound oligonucleotide, the molecular mass of the band is 87 kDa. A very faint signal, possibly caused by insoluble materials, was seen in the interface of the stacking gel and the separating gel.

Reference

1. Nakano K , B.E.A.M.V.K. A ribonucleotide reductase gene is a transcriptional target of p53 and p73. Oncogene, *19*: 4283-4289, 2000.

2. Xu, D., Wang, Q., Gruber, A., Bjorkholm, M., Chen, Z., Zaid, A., Selivanova , G., Peterson, C., Wiman KG, and Pisa P Downregulation of telemerase reverse transcriptase mRNA expression by wild type p53 in human tumor cells. Oncogene, *19*: 5123-5133, 2000.

3. Kao, S., Lemoine, F., and Marriott SJ Suppresssion of DNA repair by human T cell leukemia virus type 1 Tax is rescued by a functional p53 signaling pathway. J Biol Chem, *275*: 35926-35931, 2000.

4. Zhu, Q., Wani , M., El-Mahdy M, Wani, G., and Wanni, A. Modulation of transcriptional activity of p53 by ultraviolet radiation : linkage between p53 pathway and DNA repair through damage recognition. Mol.Carcinog., *28*: 215-224, 2000.

5. Hirose , Y., Berger, M., and Pieper, R. p53 effects both the duration of G2/M arrest and the fate of temozolomide-treated human glioblastoma cells. Cancer Research, *61*: 1957-1963, 2001.

6. Jeffy , B., CHen, E., Gudas, J., and Romagnolo, D. Disruption of cell cycle kinetics by bezo(a)pyrene: inverse expression patterns of BRCA-1 and p53 in MCF-7 cells arrested in S and G2. Neoplasia, *2*: 460-470, 2000.

7. Seluanov, A., Gorbunova, V., FAlcovitz, A., Sigal, A., Milyavsky, M., Zurer, I., Shohat, G., and Goldfinger N, R., V Changes of the death pathway in senescent human fibroblasts in response to DNA damage is caused by an inability to stablized p53. Mol Cell Bio, *21*: 1552-1564, 2001.

8. Peeper, D., Dannenberg, J.D.S., te Riele H, and Bernards, R. Escape from premature senescence is not sufficient for oncogenic transformation by Ras. Nat Cell Biol, *3*: 198-203, 2001.

9. Zeng, X., Keller, D., Wu, L., and Lu, H. UV but not gamma irradiation accelerates p53-induced apoptosis of teratocarcinoma cells by repressing MDM2 transcription. Cancer Research, *60*: 6184-6188, 2000.

10. Uberti, D. and Grilli, M. Induction of p53 in the glutamate-induced cell death program. Amino Acids, *19*: 2532000.

11. Ito, A., Lai, C., Zhao, X., Saito, S., Hamilton, M., Appella, E., and Yao, T. p300/CBP-mediated p53 acetylation is commonly induced by p53 activating agents and inhibited by MDM2. EMBO J., *20*: 1331-1340, 2001.

12. Wang, X., Ongkeko, W., Lau , A., Leung, K., and POON, R. Apossible role of p73 on the modulation of p53 level through MDM2. Cancer Research, *61*: 1598-1603, 2001.

13. Wang, J. and Friedman, E. Downregulation of p53 by sustained JNK activation during apoptosis. Mol.Carcinog., *29*: 179-188, 2000.

14. Mokdad-Gargouri, R., Belhadj, K., and Gargouri , A. Transcriptional control of human p53 expression in yeast mediated by 5'-UTR-ORF structural interaction. Nucleic Acids Res, *29*: 1222-1227, 2001.

15. Lee, S. and Rho, H. Transcriptional repression of the human p53 gene by hepatitis B viral X protein. Oncogene, *19*: 468-471, 2000.

16. Benoit, V., Hellin, A., Huygen, S., Gielen, J., Bours, V., and Merville, M. Additive effect between NF-kappaB subunits and p53 protein for transcritional activation of human p53 promoter. Oncogene, *19*: 4787-4794, 2000.

17. Nayak, B. and Das, B. Differential binding of NF1 transcription factor to p53 gene promoter and depletion in human breast tumours. Mol Biol Rep, *26*: 223-230, 1999.

18. Lee, M., Song, H., Yu, S., Lee, K., and Park, J. A 40-kDa NF1-like protein, not YY1, binds to the rat p53 promoter for transactivation in various rat organs. Biochem Cell Biol, *77*: 209-214, 1999.

19. Lee, M., Song, H., Park, S., and Park, J. Transcription of the rat p53 gene is mediated by factor binding to two recognition motifs of NF-1like protein. Biol Chem, *379*: 1333-1340, 1998.

20. Hans-Christoph kirch, Susanne, F., Heike, R., Dieter, B., and Helmut, E. Expression of human p53 requires synergistic activation of transcription from the p53 promoter by AP-1, NF-KB and Myc/Max. Oncogene, *18*: 2728-2738, 1999.

21. Reich, N.C. and Levine, A.J. Growth regulation of a cellular tumor antigen, p53, in non-transfermed cells. Nature, *308*: 199-201, 1984.

22. Soini, Y., Kamel D, Nuorva, K., Lane DP, and Vahakangsa, K.P.P.A.A. Low p53 protein expression in salivary gland tumors compared with lung carcinomas. Pathol Anat Histopatho, *421*: 415-420, 1992.

23. Balint, E. and Reisman, D. Increased rate of transcription contributes to elevated expression of the mutant p53 gene in Burkitt's lymphoma cells. Cancer Research, *56*: 1648-1653, 1996.

24. Pei, X., Nakanish , Y., Takayama, K., Bai, F., and Hara, N. Benzole(a)pyrene activates the human p53 gene through induction of nuclear factor NF-kB activity. J.Biol.Chem., *274*: 35240-35246, 1999.

25. Nayak, B. and Das, B. differential binding of nuclear proteins to theTP53 gene promoter in male breast tumours. FASEB J, *33*: 1484-1487, 1997.

26. Raman, V., Martensaen, S., Reisman, D., Evron, E., Odenwald, W., Faffee, M.J., and Sukumar, S. Compromised HOXA5 function can limit p53 expression in human breast tumours. Nature, *405*: 974-978, 2000.

27. Noda, A., Toma-Aiba, Y., and Fujiwaba, Y. A unique, short sequence determins p53 gene basal and UV-inducible expression in normal human cells. Oncogene, *19*: 21-31, 2000.

28. Zarling, J.M., Shoyab, M., Marquardt, H., Hanson, M.B., Lionbin, M.N., and Todaro, G.J. Oncostatin M: a growth regulator produced by differentiated lymphoma cells. Proc.Natl.Acad.Sci.USA., *83*: 9739-9743, 1986.

29. Horn, D., Fitzpatrick, W.C., Gompper, P.T., Ochs, V., Bolton-Hanson, M., Zarling, J.M., Malik, N., Todaro, G.J., and Linsley, P.S. Regulation of cell growth by recombinant oncostatin M. Growth Factors, *2*: 157-165, 1990.

30. Liu, J., Spence, M.J., Wallace, P.M., Forcier, K., Hellstrom, I., and Vestal, R.E. Oncostatin M-specific receptor mediates inhibition of breast cancer cell growth and down-regulation of the c-myc proto-oncogene. Cell Growth.&Differ., *8*: 667-676, 1997.

31. Spence, M.J., Vestal, R.E., and Liu, J. Oncostatin M-mediated transcriptional suppression of the c-myc gene in breast cancer cells. Cancer Research, *57*: 2223-2228, 1997.

32. Douglas, A.M., Goss, G.A., Sutherland, R.L., Hilton, D.J., Berndt, M.C., Nicola, N.A., and Begley, C.G. Expression and function of members of the cytokine receptor superfamily on breast cancer cells. Oncogene, *14*: 661-669, 1997.

33. Douglas, A.M., Grant, S.L., Goss, G.A., Clouston, D.R., Sutherland, R.L., and Begley, C.G. Oncostatin M induces the differentiation of breast cancer cells. Int.J.Cancer, *75*: 64-73, 1998.

34. Liu, J., Li, C., Ahlborn, T.E., Spence, M.J., Meng, L., and Boxer, L.M. The expression of p53 tumor suppressor gene in breast cancer cells is down-regulated by cytokine oncostatin M. Cell Growth.&Differ., *5*: 15-18, 1999.

35. Huang, L., Sowa, Y., Sakai, T., and Bpardee, A. Activation of the p21wafl/CIP1 promoter independent of p53 by the histone deacetylase inhibitor suberoylanilide hydroxamic acid(SAHA) through the Sp1 sites. Oncogene, *19*: 5712-5719, 2000.

36. Briggs, J., Kudlow, J., and Kraft, A. The role of the transcription factor Sp1 in regulating the expression of the WAF1/Cip1 gene in U937 leukemic cells. J.Biol.Chem., *271*: 901-906, 1996.

37. Datto, M., Yu, Y., and Wang, X. Functional analysis of the transforming growth factor beta responsive elements in the WAF1/Cip1/P21 promoter. J.Biol.Chem., *270*: 28623-28628, 1995.

38. Nakano, K., Mizuno, T., Sowa, Y., Orita, T., Okuyama, Y., Fujita, T., Ohtani, F., Matsukawa, Y., and Tokino, T. Butyrate activates the WAF1/Cip1 gene promoter through Sp1 site in a p53-negative human colon cancer cell line. J.Biol.Chem., *272*: 22199-22206, 1997.

39. Sowa, Y., Orita, T., minamikawa, S., Nakano, K., Mizuno, T., Normura, H., and Sakai, T. Histone deacetylase inhibitor activates the Waf1/Cip1 gene promoter through the Sp1 site. Biochem.Biophys.Rea.Comm, *241*: 142-150, 1997.

40. Liu, J., Ahlborn, T.E., Briggs, M.R., and Kraemer, F.B. Identification of a novel sterol-independent regulatory element in the human low density lipoprotein receptor promoter. J.Biol.Chem., *275*: 5214-5221, 2000.